In Vivo Comparison of Copper Blood-Pool Agents: Potential Radiopharmaceuticals for Use with Copper-62

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Two techniques for labeling of albumin with copper-67 (^{67}Cu) and ^{62}Cu were investigated; one using the native Cu(II) binding site of the protein and the other employing a bifunctional chelate, 6-bromoacetamidobenzyl-1,4,8,11-tetraazacyclotetradecane-N,N′N″,N‴-tetraacetic acid (Br-benzyl-TETA or BAT), conjugated to the protein. Rat biodistribution experiments with ^{67}Cu demonstrated retention of i.v. ^{67}Cu-benzyl-TETA-albumin in the blood pool identical to co-injected ^{125}I-albumin. By contrast, i.v. administration of either [^{67}Cu]-Cu-acetate or [^{67}Cu]-Cu-acetate pre-mixed with albumin results in relatively rapid clearance of blood-pool radioactivity as the tracer is excreted into the urine. The ^{62}Cu-benzyl-TETA-albumin radiopharmaceutical was obtained in ca. 17% radiochemical yield (end of synthesis, without decay correction) following a procedure that can be completed in 15–18 min. In PET experiments with a baboon, myocardial blood volume images with ^{62}Cu-benzyl-TETA-albumin were identical to those obtained with C^{15}O. Use of the ^{62}Cu-benzyl-TETA-albumin image for blood-pool subtraction of a ^{62}Cu-PTSM myocardial perfusion image is illustrated. Copper-62-benzyl-TETA-HSA should be a useful, generator-produced radiotracer for the detection of the vascular pool at PET facilities without cyclotrons.

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The zinc-62/copper-62 (^{62}Zn/^{62}Cu) radionuclide generator system is a potential source of positron emitting radiotracers for PET imaging facilities that lack an in-house cyclotron. We have recently described the preparation and performance of a high-level (>300 mCi) ^{62}Cu generator system, and the synthesis and evaluation of ^{62}Cu-PTSM (pyruvaldehyde bis(N^4-methylthiosemicarbazonato)copper(II)) as a PET tracer for cerebral and myocardial perfusion (*1–3*). To complement the information available from a PET perfusion study with ^{62}Cu-PTSM, it may be desirable to have available a ^{62}Cu-radiopharmaceutical for delineation of blood volume. Such an agent should be useful for identification of vascular structures; for use with tracers that need to be corrected for radioactivity emanating from the vascular space; and potentially, for estimation of regional leakage of protein from the vascular space into tissue (as may occur with ischemia followed by reperfusion). When used with gated data acquisition, blood-pool agents can also be used for assessment of ventricular function during a PET study (*4*).

Albumin is the most abundant plasma protein and in most species contains a high-affinity binding site for the transport of the Cu(II) ion (*5–6*) [log K = 16.2 (*7*)]. Thus, it may be feasible to directly label albumin with ^{62}Cu by ligand exchange using a weakly coordinating buffer solution as the source of ^{62}Cu(II) ion. Alternatively, labeling of albumin with ^{62}Cu could be accomplished using a bifunctional chelate (with affinity for copper) to covalently link the ^{62}Cu to the protein. Several laboratories have worked on the development of bifunctional chelates for labeling antibodies with ^{67}Cu (*8–13*). One of the most effective is 6-bromoacetamidobenzyl-1,4,8,11-tetraazacyclotetradecane-N,N′,N″,N‴-tetraacetic acid (Br-benzyl-TETA or BAT) (*12*).

We report here the preparation of benzyl-TETA-albumin (also abbreviated as HSA-2IT-BAT); the radiolabeling of this produce with both ^{67}Cu and ^{62}Cu, a comparison of the rat plasma clearance of ^{67}Cu-benzyl-TETA-albumin, [^{67}Cu]-Cu-acetate, and ^{125}I-HSA; and PET studies in a baboon to compare ^{62}Cu-benzyl-TETA-HSA and ^{15}O-carboxyhemoglobin as tracers for regional myocardial blood volume.

MATERIALS AND METHODS

Human serum albumin (HSA), rat albumin, 2-iminothiolane, beta-mercaptoethanol, iodoacetiamide, triethanolamine,

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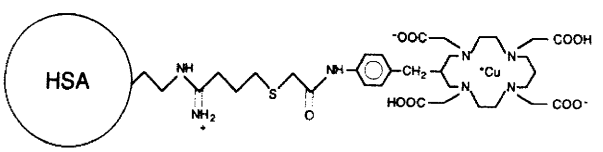
sodium chloride, sodium citrate, sodium phosphate, and Sephadex® G-25 were obtained from Sigma Chemical Co., St. Louis, MO and used without further purification. Distilled, deionized water (Milli Q®; >17MΩ/cm resistivity) was used for preparing buffers and reagents. Puratronic grade HCl and sodium acetate were obtained from Johnson Matthey. Centrifree® protein membrane separation apparatus were obtained from Amicon Corp. and Millex GV_4 low-protein binding 0.22-μm filters were obtained from Millipore Corp. Chelex 100 25-mm sample preparation discs were purchased from Bio-Rad Laboratories. Sephadex G-25 in 150 m*M* sodium phosphate, pH 8, was used to prepare 1-ml and 3-ml spin columns as described elsewhere (*14*). 6-Br-benzyl-TETA was prepared as described by Moi et al. (*10,12*). Iodine-125-sodium iodide (363 mCi/ml in 0.1 *M* NaOH) was purchased from E.I. DuPont de Nemours (Boston, MA). Copper-67 (1 mCi/6 ml in 1N HCl) was obtained from the Missouri University Research Reactor (Columbia, MO). Zinc-62/copper-62 generators were prepared as previously described from which ^{62}Cu can be efficiently eluted (30–200 mCi/1–3 ml, dependent on the age of the generator) (*1*). Production of $H_2{}^{15}O$ and $C^{15}O$ was as described previously (*15*).

Copper-67-labeled-benzyl-TETA-albumin (Fig. 1). Radiolabeled albumin was prepared by evaporating the ^{67}Cu (in 1 N HCl) to dryness and reconstituting in 50 μl of 1 N HCl. To the ^{67}Cu solution, 200 μl of 0.4 *M* acetate (pH 5) was added. The rat albumin and HSA were conjugated with Br-benzyl-TETA as described elsewhere (*11,12*) and stored frozen (−80°C) in 1-mg aliquots. One milligram of benzyl-TETA-albumin (in ~150 μl of 150 m*M* sodium phosphate, pH 8) was thawed and mixed with [^{67}Cu]-Cu-acetate (pH 3–4). After 5–30 min, the protein solution was purified on a 1-ml Sephadex G-25 spin column (*14*) (in 150 m*M* sodium phosphate, pH 8). Control samples of [^{67}Cu]-Cu-acetate and [^{67}Cu]-Cu-acetate mixed with albumin were also prepared and applied to equivalent gel columns.

Iodine-125-labeled-HSA. HSA was prepared with iodogen as described elsewhere (*16*) and purified by separation on a Sephadex G-25 spin column.

Copper-62-labeled-benzyl-TETA-albumin. Radiolabeled albumin was prepared by eluting ^{62}Cu from a $^{62}Zn/^{62}Cu$ generator in 1–4 ml of 2 N HCl. Two equivalents of sodium acetate were added and mixed. The ^{62}Cu-acetate was passed through a pretreated Chelex 100 sample preparation disc, which was further washed with 40 ml of 0.002 N HCl. The disc was then eluted with 2 N HCl and 0.2 ml fractions were collected. To one or two fractions, which contained the majority (>85%) of the ^{62}Cu radioactivity, two equivalents of sodium acetate were added. The ^{62}Cu-acetate was added to a thawed, 1-mg sample of benzyl-TETA-HSA. After a 5-min incubation at room temparature, the conjugated-albumin solution was purified on a Sephadex G-25 spin column. The entire preparation required 15–18 min (from beginning of elution) and proceeded with 16.6% ± 1.4% radiochemical yield (end of synthesis, without decay correction).



*Cu-Benzyl-TETA-HSA

FIGURE 1
Scheme of Cu-Benzyl-TETA-HSA.

Tracer Biodistribution. Distribution was determined in anesthetized adult (175–225 g) female Sprague Dawley rats. Copper-67-labeled-benzyl-TETA-HSA or ^{67}Cu-labeled-benzyl-TETA-rat albumin was coinjected (intravenously via exposed femoral vein) with ^{125}I-HSA. Copper-67-Cu-acetate and [^{67}Cu]-Cu-acetate premixed with rat albumin (purified by a Sephadex G-25 spin column) were also evaluated. After the appropriate time (1–60 min), the rat was reanesthetized and a heparinized blood sample removed by direct cardiac puncture. The removed blood samples were centrifuged (15,000 × g for 2 min) and the plasma removed. Both the plasma and the packed red blood cells were counted in an automated well-type NaI(Tl) gamma scintillation counter. The anesthetized rats were killed by decapitation 60 min after injection and the following tissues were dissected, weighed, and counted: blood, lung, liver, spleen, kidney, bladder, muscle, fat, heart, and brain.

Anti-rat Albumin Affinity Chromatography. Chromatography was performed on a 1 × 20-cm CNBr-activated Sepharose 4B (Sigma Chemical Co.) column coupled with rabbit anti-rat-albumin (IgG fraction, Organon Teknika Corp., Cappel Research Reagents) (*17–18*). The column was eluted at 0.4 ml/min, first with 0.1 *M* sodium phosphate, 0.1 *M* sodium citrate and 0.25 *M* sodium chloride pH adjusted to 7.25. After elution of 25 ml, the column was switched to the same buffer composition with the pH adjusted to pH 2.50. The samples were collected in 1.75-ml fractions and counted to determine the distribution of radioactivity. Copper-67-benzyl-TETA-rat albumin was evaluated, as well as ^{62}Cu-benzyl-TETA-HSA.

FPLC of ^{62}Cu-benzyl-TETA-HSA. Fast protein liquid chromatography (FPLC) was done with a Pharmacia/LKB chromotograph with a Superpose 6 size exclusion column eluted at 0.5 ml/min with 0.9% NaCl solution. The eluate was monitored for UV absorption at 254 nm and then collected in 2-min fractions to determine the radioactivity concentration per fraction. The ^{62}Cu-benzyl-TETA-HSA, the unlabeled-benzyl-TETA-HSA, and the unconjugated HSA (1 mg/0.2 ml of 150 mm sodium phosphate, pH 8) were chromatographed.

HPLC of ^{62}Cu-benzyl-TETA-HSA. A Shodex Protein WS series 0.9 × 30-cm column (Showa Denko K.K.) eluted with water at 1 ml/min was used for high-performance liquid chromatography (HPLC). The eluate was continuously monitored for UV absorbance (280 nm) and radioactivity with a shielded NaI(Tl) detector (2 × 2 in).

Baboon PET Imaging. An adult male baboon (23 kg) was pretreated with 0.2 mg atropine i.m. and sedated with ketamine (25 mg/kg i.m.), and intubated and ventilated with 70% nitrous oxide/30% oxygen after paralysis was induced with gallamine (ca. 1.7 mg/kg i.v.). Arterial blood gases (pH, pCO_2, pO_2), end-tidal pCO_2, and arterial blood pressure were monitored. An 18-g plastic catheter (Quickcath, 8.7 cm) was placed percutaneously in the femoral artery for blood sampling and physiologic monitoring. Additionally, an 18-g plastic catheter was situated in a peripheral vein for bolus i.v. injections, fluid replacement, and supplemental drug administration. The ba-

boon was aligned in Super PETT IIB (a time-of-flight, seven-slice positron emission transaxial tomograph (PETT) with a 50-cm diameter field) in a supine position so that the heart was centered within the 10.2-cm axial field of view.

$C^{15}O$ (20 mCi) was administered by manual ventilation with the radioactive gas. After allowing time (1 min) for clearance of ^{15}O from the lungs and equilibration of tracer in the blood pool, data were collected for 5 min. After the ^{15}O had decayed, $H_2{}^{15}O$ (14.4 mCi) was injected as a bolus via the peripheral vein catheter. Data were collected for 150 sec beginning at the time of injection. The ^{15}O was allowed to decay and 8 mCi of ^{62}Cu-benzyl-TETA-HSA was injected (i.v.). Data were collected for 20 min starting 30 sec after tracer administration. Then, after allowing for decay of the administered ^{62}Cu to background levels, ^{62}Cu-PTSM (14 mCi) was injected (i.v.) and data collected for 20 min. Images were reconstructed from list-mode data. Timed with each injection of $H_2{}^{15}O$ and ^{62}Cu-PTSM, rapid arterial blood samples (every 2–5 sec) were collected in preweighed 1-ml capped syringes. The blood samples were weighed and counted in a calibrated NaI(Tl) scintillation well detector. The counts in the blood samples were decay-corrected to the beginning of PET collection. Blood samples were withdrawn at 5-min intervals after ^{62}Cu-benzyl-TETA-HSA injection to determine protein binding and in vivo stability.

RESULTS

In the initial experiment with longer-lived ^{67}Cu ($t_{1/2}$ = 59 hr), ^{67}Cu-benzyl-TETA-albumin was obtained with a *59.8% ± 12.4% (n = 5) radiochemical yield after a 5-min incubation at room temperature and purification* on a Sephadex G-25 spin column. The ^{67}Cu-benzyl-TETA-albumin retained 89.7% ± 9.3% of its physiologic properties as measured by anti-albumin CNBr-Sepharose 4B affinity chromatography. Copper-67-Cu-acetate mixed with albumin under the same conditions had ca. 1% of the radioactivity associated with the albumin. Copper-67-Cu-acetate alone did not elute from the Sephadex G-25 spin column. Iodine-125-HSA was efficiently radiolabeled with ca. 40% radiochemical yield after purification on a Sephadex-G-25 spin column. Copper-62-benzyl-TETA-HSA was efficiently synthesized with a high purity final product (99 + % by HPLC, Fig. 2) in sufficient quantity for use in PET imaging studies (Table 1). FPLC of ^{62}Cu-benzyl-TETA-HSA indicates that the radioactivity is associated with the major protein fractions that elute at ca. 26 min after injection. This FPLC fraction would contain proteins with molecular weight of ca. 68,000.

Rat biodistribution data for ^{67}Cu-benzyl-TETA-HSA [^{67}Cu]-Cu-acetate, ^{125}I-HSA, and [^{67}Cu]-Cu-acetate premixed with albumin are present in Table 2. Copper-67-benzyl-TETA-rat albumin was also evaluated with virtually identical results. The amount of radioactivity (% ID/g) in heparinized blood samples withdrawn between 1 and 60 min after injection was determined (Fig. 3). The blood clearance of ^{67}Cu-benzyl-TETA-HSA and ^{125}I-HSA are the same ($0.5 > p > 0.1$); however, the

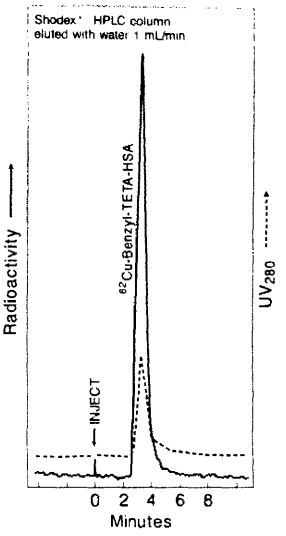


FIGURE 2
HPLC chromatogram for ^{62}Cu-benzyl-TETA-HSA on a Shodex WS series (0.9 × 30 cm) size exclusion column eluted with water at 1 ml/min. The continuously monitored radioactivity (– – –) and the UV absorbance (- - - -) are plotted from injection (t = 0). Both peaks were exclusively detected at 3.6 min.

[^{67}Cu]-Cu-acetate and [^{67}Cu]-Cu-acetate premixed with HSA clear rapidly from the blood as the tracer is excreted into the urine (Fig. 3). There was minimal difference between the two controls, [^{67}Cu]-Cu-acetate and [^{67}Cu]-Cu-acetate + HSA, but there were significant differences between these controls and ^{67}Cu-benzyl-TETA-HSA ($p < 0.001$) and ^{125}I-HSA ($p < 0.001$), with the latter two tracers retained in the blood for a prolonged time. It also should be noted in comparing ^{67}Cu-benzyl-TETA-HSA and ^{125}I-HSA that the concentration of the radioactivity in the plasma is not significantly different at anytime during the study (Fig. 4).

The images obtained from the baboon sequentially given $C^{15}O$, $H_2{}^{15}O$, ^{62}Cu-benzyl-TETA-HSA, and ^{62}Cu-PTSM are shown in Figure 5A–B. The ^{62}Cu-benzyl-TETA-HSA provides an image of equal quality and distribution as ^{15}O-carboxyhemoglobin (Fig. 5A). Furthermore, the ^{15}O-water myocardial perfusion image, following standard correction for $H_2{}^{15}O$ in the vascular space using $C^{15}O$, is qualitatively the same as the image created with ^{62}Cu-labeled radiopharmaceuticals (^{62}Cu-PTSM- and ^{62}Cu-benzyl-TETA-HSA) (Fig. 5B) (*18*). The blood samples withdrawn after the ^{62}Cu-benzyl-TETA-HSA was injected (1, 5, 10 minutes) had ca. 90% of the radioactivity associated with the plasma.

TABLE 1
Radiochemical Properties of ^{62}Cu-benzyl-TETA-HSA (n = 5)

Radiolabeling yield*	64.5% ± 9.2%
Preparation time	15–18 min
Radiochemical purity (HPLC)	99 + %
Affinity on anti-albumin CNBr-Sepharose 4B column	95%–99%

* % radiolabeling determined by amount ^{62}Cu with protein versus total applied to spin column and decay-corrected to a common time point.

TABLE 2
Biodistribution in Sprague Dawley Rats at 1 Hour
(%ID/g; X ± s.d.; n = 4)

	Coinjected			
	^{67}Cu-benzyl-TETA-HSA	^{125}I-HSA	[^{67}Cu]-Cu-acetate	[^{67}Cu]-Cu-acetate + HSA*
Blood	6.34 ± 0.42	5.63 ± 0.31	0.89 ± 0.087	0.73
Lung	2.25 ± 1.10	—	0.85 ± 0.51	0.84
Liver	2.37 ± 0.08	0.83 ± 0.09	2.97 ± 1.33	3.53
Spleen	1.09 ± 0.13	0.73 ± 0.16	0.31 ± 0.15	0.39
Kidney	1.21 ± 0.05	1.12 ± 0.26	6.90 ± 2.64	7.57
Bladder	1.07 ± 0.89	—	1.46 ± 1.73	0.72
Muscle	0.38 ± 0.17	0.12 ± 0.10	0.20 ± 0.11	0.18
Fat	0.21 ± 0.09	—	0.19 ± 0.08	0.19
Heart	1.02 ± 0.05	—	0.48 ± 0.18	0.47
Brain	0.15 ± 0.013	—	0.05 ± 0.02	0.05

* Due to the very low efficiency of preparing this radioactive compound, only one animal was studied.

DISCUSSION

To illustrate the possible application of a ^{62}Cu-blood-pool tracer, we synthesized and utilized ^{62}Cu-benzyl-TETA-HSA. Images obtained from the baboon heart enabled a blood-pool correction of a ^{62}Cu-PTSM image of myocardial perfusion. Figure 5B shows the ^{62}Cu-PTSM image 0–2 min after administration of tracer and the image after correction of ^{62}Cu-PTSM in the blood pool with the ^{62}Cu-benzyl-TETA-HSA image. Images correlate closely with those obtained using $H_2{}^{15}O$ after the correction for $H_2{}^{15}O$ in the vascular component with the use of ^{15}O-carbon monoxide (which binds avidly to hemoglobin). The later technique has been validated extensively and provides accurate illustration of myocardial perfusion (*19–22*). The blood-pool correction makes the ^{62}Cu-PTSM image more closely resemble the $H_2{}^{15}O$ perfusion image. However, as we have reported previously, blood-pool correction is not necessary to render reasonably high quality images of myocardial perfusion with ^{62}Cu-PTSM and PET (*1,3*).

The observation that ^{62}Cu-benzyl-TETA-HSA clears from the blood pool analogously to ^{125}I-HSA and provides PET images comparable to $C^{15}O$ images suggests that this generator-produced radiopharmaceutical can be used for delineation of the blood pool or more properly, of plasma (albumin) space. Potential uses (aside from the use to correct images obtained with other tracers for radioactivity emanating from the blood pool) include delineation of vascular structures, assessment of protein leakage from the vascular space into injured tissue, and PET assessments of ventricular func-

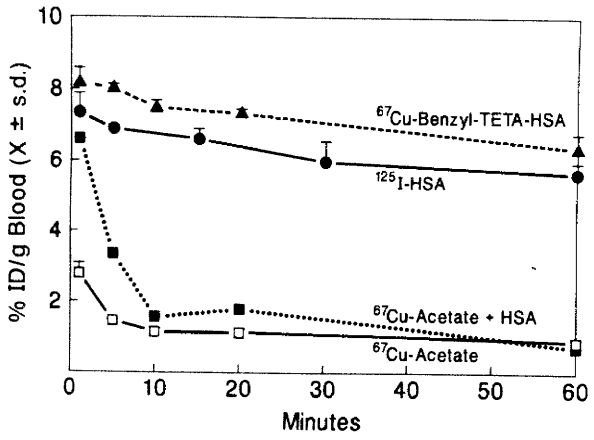


FIGURE 3
Blood clearance of the blood-pool radiotracers 1–60 min after i.v. injection in adult Sprague-Dawley rats. Notice the relatively rapid blood clearance of both [^{67}Cu]-Cu-acetate alone (□) and [^{67}Cu]-Cu-acetate premixed with HSA (■).

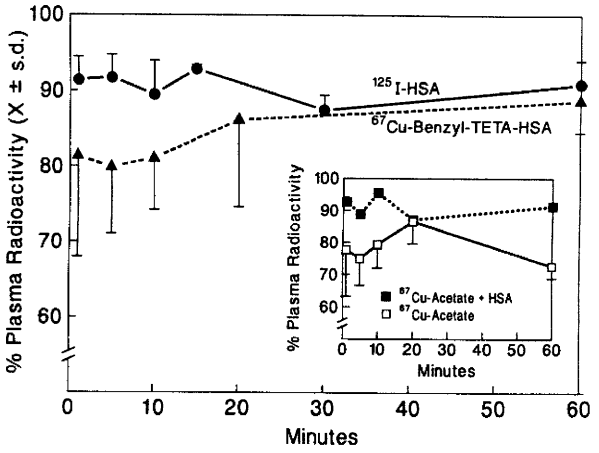


FIGURE 4
The amount of radioactivity in the plasma compared to the total blood sample (% plasma radioactivity) determined between 1 and 60 min after i.v. injection in Sprague-Dawley rats. Although the controls ([^{67}Cu]-Cu-acetate alone and [^{67}Cu]-Cu-acetate + HSA) clear the blood pool quickly (see Fig. 3), the radioactivity remaining is mostly plasma-associated.

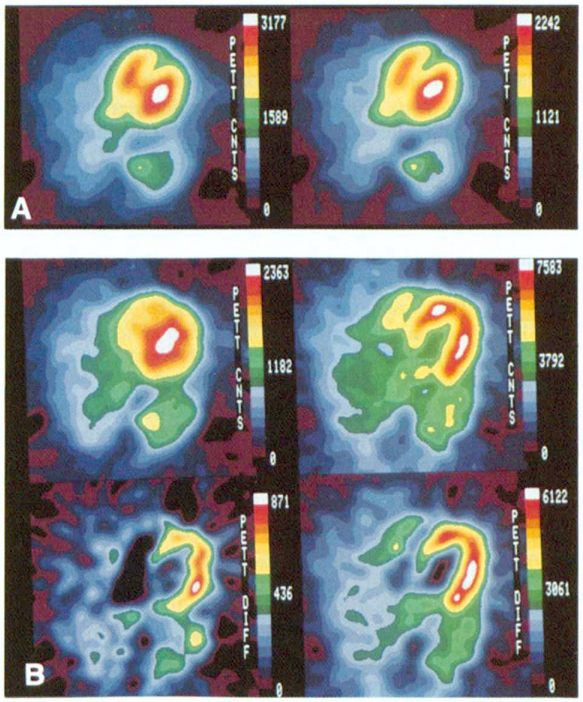


FIGURE 5
(A) PET images of the baboon heart obtained after i.v. administration of 8 mCi ^{62}Cu-benzyl-TETA-HSA (left) and the corresponding image obtained at the same midventricular level after inhalation of ca. 20 mCi of $C^{15}O$ (right). The ^{62}Cu-benzyl-TETA-HSA image was obtained from 20 min of data and contained 445,150 counts/slice. The right ventricular chamber is to the reader's left, with the left ventricular chamber to the right. Anterior is to the top and the descending aorta is seem below. (B) The PET images of the same animal in the same position after i.v. injection of $H_2{}^{15}O$ (upper left) and ^{62}Cu-PTSM (0–2 min accumulation, upper right). Both images are uncorrected for radioactivity emanating from the blood-pool activity. The standard perfusion image obtained after $H_2{}^{15}O$ was generated (lower left) by correcting the $H_2{}^{15}O$ image for radioactivity in the vasculature using a second scan obtained with $C^{15}O$ and a previously described technique (*19*). This same approach was then applied to the ^{62}Cu-PTSM data whereby the data obtained was corrected for radioactivity in the blood pool using information from the ^{62}Cu-benzyl-TETA-HSA scan. The resultant image is depicted at lower right.

tion when data from the heart is collected in conjunction with cardiac gating (*4*).

Yokoyama et al. (*23–26*) have reported the ^{62}Cu labeling of a *bis*(thiosemicarbazone) bifunctional chelate attached to albumin, but the details necessary for a direct comparison with ^{62}Cu-benzyl-TETA-HSA are absent. However, since benzyl-TETA-conjugated proteins have been shown to possess a remarkably high affinity for Cu(II) and excellent stability in serum (*27–29*), it seems unlikely that the *bis*(thiosemicarbazone)-albumin product prepared by Yokoyama et al. would be superior to benzyl-TETA-HSA. It should perhaps be noted that images obtained with ^{62}Cu-acetate may also accurately reflect blood-pool distribution in the chest. Excluding the excretory organs (liver and kidneys) from consideration, the organ-specific uptake of ionic copper will be negligible over a time frame relevant to ^{62}Cu-imaging (Reference 30 and Table 2), while the tracer remaining in the vascular space will be protein bound (*6*). Although the dynamics of the plasma clearance of ^{62}Cu-acetate would substantially reduce the image counts and increase the radiation dose to the excretory organs, compared to an equivalent dose of ^{62}Cu-benzyl-TETA-HSA, the simplicity of the former may make it worthy of further investigation.

CONCLUSIONS

While in the absence of an in-house cyclotron PET blood volume images can be obtained with a generator-produced radiotracer, ^{68}Ga-transferrin. The 68-min half-life of gallium may pose a problem in imaging protocols that involve multiple radiotracer injections. Copper-62-benzyl-TETA-HSA provides an alternative generator-produced blood-pool tracer with nearly ideal properties. This study demonstrates that ^{62}Cu-benzyl-TETA-HSA is an effective generator-produced positron-emitting radiotracer for imaging blood pool with PET, since the 9.7-min half-life of ^{62}Cu lends itself to multi-injection imaging protocols. From the standpoint of radiation dosimetry, if one compares ^{62}Cu and ^{68}Ga tracers that are both completely retained in the vascular space, the patient radiation exposure per millicurie ^{62}Cu administered will be approximately five-fold lower than the exposure per mCi of ^{68}Ga (*31*). In a PET center remote from a cyclotron, the ^{62}Zn/^{62}Cu generator could serve as a radionuclide source for preparation of both a multi-organ perfusion tracer (^{62}Cu-PTSM) and a blood volume tracer (^{62}Cu-benzyl-TETA-HSA).

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EDITORIAL
Cardiac Blood-Pool Tracers

Today, cardiac imaging can be performed with a scintillation camera on line with a computer by single-photon emission computed tomography (SPECT) or by positron emission tomography (PET) using suitable radiopharmaceuticals. While PET blood-pool imaging is not a common procedure, the enhanced resolution of PET and its ability to provide tomographic delineation of the cardiac structures has the potential to improve the quality of diagnostic information that can be gleaned from this study.

Blood-pool imaging began in 1958 (*1*) with radioiodinated (^{131}I) human serum albumin for the detection of pericardial effusion. In the

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